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## Year 2 Annual Report

Grant #: IRP 95-101

Title: Quantitation of Bone Growth Rate Variability in Rats Exposed to Micro- (near zero G) and Macrogravity (2G)

Investigators:

Timothy G. Bromage (Hunter College)  
Stephen B. Doty (Hospital for Special Surgery)  
Igor Smolyar (Hunter College)  
Emily Holton (NASA)

## INTRODUCTION

### Aims

Our stated primary objective is to quantify the growth rate variability of rat lamellar bone exposed to micro- (near zero G: e.g. Cosmos 1887 & 2044; SLS-1 & SLS-2) and macrogravity (2G). The primary significance of the proposed work is that an elegant method will be established that unequivocally characterizes the morphological consequences of gravitational factors on developing bone. The integrity of this objective depends upon our successful preparation of thin sections suitable for imaging individual bone lamellae, and our imaging and quantitation of growth rate variability in populations of lamellae from individual bone samples.

### Year 1: Recap

Our initial efforts were to be spent on specimen preparation as NASA had hoped that our equipment vendor would supply us despite the restructuring of our 2 year program into 3 years. While our vendor could not comply, we nevertheless prepared less sophisticated sections that could still satisfy the simpler objectives of Year 1. We were very successful, documenting the lamella formation rate for the SLS experimental rat. The most astonishing result was that endosteal lamellae of the humerus (and we expect in many other locations) of the juvenile rat accrue at the rate of 1 bone lamella per day (Figure 1). In other words, the lamella formation rate follows a circadian rhythm. This is the first calculation of a lamellar formation rate in the mineralized tissue sciences.

We also made preliminary observations suggesting that the vital labels, calcein and demeclocycline, used in SLS experimental protocols, have a negative effect on bone formation rates.

Given the delay in refined specimen preparation, we worked hard in other ways to prepare for the quantitative aspect of our research to begin in Year 2: we configured the series of image processing steps that provide binary "working" images for growth rate quantification, and we prepared a preliminary version of a Windows®-based program developed for automating the

measurement taking and for configuring a 3-D chart illustrating bone growth rate variability with precision to 24 hours.

## Year 2: Expectations

During the first half of Year 2 we explained that quantitative analyses of lamellar growth rate variability of SLS-2 flight bone and their controls would begin. Backscattered electron microscopy in the scanning electron microscope (BSE-SEM) of SLS-1 and SLS-2 bone specimens would be performed in order to assess mineral density changes owing to microgravity.

Quantitative investigations of lamellar growth rate variability would begin on Cosmos 1887 and 2044 samples during the second half of Year 2, as would the accompanying BSE-SEM of these bone specimens.

The results of our investigations on the morphological and mineral density changes affecting the development of lamellae under conditions of microgravity were to be summarized, written up in more final form for publication, and for presentation to the annual meeting of the *American Society for Gravitational and Space Biology*.

## Year 2: Actual

We acquired the necessary equipment and developed our proposed specimen preparation protocol. This novel protocol permits, for the first time, the same specimen to be employed in analyses by both conventional light microscopy and scanning electron microscopy. The protocol is so important and unique that we formally presented this technique to annual meetings of *Scanning* 97 (*Scanning* 19(3):179-180, 1997) and the *American Society for Bone and Mineral Research* (*J. Bone Min. Res.* 12(1):S202, 1997).

Having readied our specimen preparation protocol for proposed quantitative analyses of lamellar growth rate variability, we were then able to assess and prepare all SLS-1 flight and control bone (Figures 1-4). All SLS-2 bone has been prepared and is currently under investigation. Preliminary BSE-SEM investigations of SLS-1 flight bone have also been performed (Figure 5).

We produced a penultimate version of our Windows®-based program (refining of this program is still underway) for automating the quantitation of lamellar growth rate variability. This unique program and the results obtained on the effects of microgravity on bone development were presented to the annual meeting of the *American Association of Physical Anthropologists* (*Am. J. Phys. Anthropol. Suppl.* 24:83, 1997).

Our cumulative results have been submitted for presentation to the annual meeting of the *American Society for Gravitational and Space Biology* (ASGSB) to be held this November in Washington, D.C. Our presentation to the ASGSB and the forthcoming publication will include results arising from our continued work on SLS-2 lamellar growth rate variability and the BSE-SEM of flight and control bone.

## MATERIALS AND METHODS

### Animals

Specimens from SLS-1 experiments are reported here:

#### Experiment: SLS-1

Experimental groups: 19 RAHF Flight and Vivarium Control

Mission Length (ML): 9 day flight (ML=9)

Species: male Harlan (Sprague/Dawley)

Bone Element: labeled humerus

The experimental group had a proscribed vital labeling regime of calcein and demeclocycline prior to and around Launch, and at Recovery (see Figure 1 for details).

### Specimen Preparation

Sections ground from methyl methacrylate embedded samples to ca. 120 microns of section thickness on a Buehler Petro-Thin (Buehler Ltd., IL) were carefully made superficially anorganic with Tergazyme (Alconox, NJ) enzyme detergent at 50° C for 24 hours. Sections were subsequently ground on one side through graded emery papers to 1200 grit on a Handimet II. The embedded section was placed onto a thin film of acetonitrile for 5 minutes. A histological slide was etched with 20% hydrofluoric acid for 4 minutes, thoroughly rinsed, and air dried.

The section was mounted onto the etched slide using the All-Bond 2 Universal Dental Adhesive System (Bisco, IL). The slide was first treated with Silane Porcelain Primer for 30 seconds and air dried. A 50:50 mixture of proprietary Primers A and B was brushed onto the polished section five coats in succession, allowed to dry, and then polymerized for 30 seconds using a visible light curing gun. The section was then pressed onto the slide, the 1200 grit side face down, in a small pool of Dentine/Enamel Bonding Resin and light cured for 1-2 minutes.

The mounted section was again ground on 1200 grit emery paper and polished with a Buehler Ecomet III to 1.0 micron diamond and to 0.05 micron alumina slurry on a Buehler Vibromet II Vibratory Polisher until reaching ca. 100 microns of section thickness. Save for the embedding and vibratory polishing procedures, the method outlined above requires only 30 minutes of specimen handling time versus 1-2 days using more traditional methods.

The embedded polished thin section was coverslipped with 100% ethyl acetate and imaged by polarizing and fluorescence microscopy. Subsequent to this the section was made electrically conductive with carbon and imaged by BSE-SEM.

### Instruments

Images of endosteal lamellae were obtained with a Leica DMRXAE light microscope fitted with linear polarizing filters and a 100 Watt fluorescence system. Images were retrieved by a Kodak Megaplus CCD camera and transported to the Leica Quantimet 600, a framestore-based

image analysis system. An image processing program was written which standardized gray level image enhancement and filtering procedures. The resulting images were then passed to the quantification program for analysis (below). The same fields of view were also imaged by a LEO S440 SEM in BSE-SEM imaging mode at 20 kV, 500 pa, and a 15 mm working distance.

## Quantification Software

We developed a Windows®-based program (C compiler for DOS environment) for processing lamellar bone. The program, titled LAMELLA, converts images of the widths between lamellae into a numerical table. The table is represented graphically in the form of a 3-D chart. The chart illustrates the quantitative results based on a Boolean function that was proposed and described in our original proposal.

The input for this software is a binary image of bone acquired from our Quantimet 600 image processing routine. This binary is transferred to LAMELLA which overlays "N" number of specified transects onto the screen image. LAMELLA automatically interacts with the binary, labeling each lamella. Measurements between each successive lamella are automatically recorded and presented in a numerical Table Q. The SURFER (Golden Software, Inc.) program is employed to represent Table Q in the form of a 3-D chart of the bone growth rate indicating growth rate in the Z direction, time in the X direction, and sampling error in the Y direction (increasing error in the 3-D chart is represented toward the rear of the chart. Charts illustrated in this report indicate very little error, however, as the initial images are rather good, and so the 3-D landscape appears rather uniform from front to back).

## ANALYSES

### Effect of Space Flight on Bone Deposition

Protocol. The experimental protocol is illustrated in Figure 1. The polarized light image at top is the source image from which the binary is created. The fluorescence image at bottom is annotated with dates of significance to the 1991 SLS-1 mission, including dates of administration of vital labels, Launch, and Recovery. An extract of the binary image is also overlain on this image, providing an account of the circadian rhythm of lamella formation discovered in Year 1 of our project.

Flight. An example of the response of bone to microgravity is presented in Figure 2. The polarized light image is at top left. Lamellar processing of this image produces the binary image against which time markers derived from the fluorescence image (particularly the dates of administration of calcein that are well defined) may be well positioned. The binary image is then subject to LAMELLA quantitative processing and SURFER to produce a 3-D output (as described above).

The 3-D chart describes the lamellar growth rate. Positions of the calcein and demeclocycline labels are shown by green and blue bands, respectively, and Launch and Recovery are indicated for reference. Growth rate is given by the Z (height) axis and time proceeds from left to right on the X

axis. Sampling error would be revealed by increasing irregularity of the chart in the Y axis from front to back, but as the data is very isotropic, little change in each 2-D profile exists along this axis. There are at first marked changes in the growth rate between the first calcein label and Launch. These results continue to suggest that the administration of the vital labels, calcein and demeclocycline, hinders osteoblastic activity. Labels are associated with short-term downward trends in the Z direction followed by recovery in the growth rates.

At Launch the 3-D chart reveals an immediate drop in bone deposition owing, perhaps, to the stress of liftoff and initial weightlessness. This is followed by a downward trend until Recovery. Recovery is also preceded by a precipitous drop in the growth rate due to calcein toxicity. This drop is followed by an increase in the growth rate at 1G until euthanized.

Control. An example of the response of bone subject to the labeling protocol at 1G is presented in Figure 3 (this figure is represented in the same way as that described for Figure 2, above). The growth rate is intermittently compromised by the labeling protocol until just prior to the Launch date. In contrast to flight conditions, the control bone reveals an increase in the lamellar growth rate following the Launch date except for a transitory drop owing to calcein toxicity.

Bone Growth Rate Summary. Figure 4 summarizes the average condition identified from the SLS-1 experimental sample. The 3-D charts clearly demonstrate that the bone growth rate is compromised in flight compared to ground controls. Previous gravitational research on the rat has lacked a certain precision regarding the morphology and character of that specific bone volume formed during spaceflight as separate from that bone volume laid down prior to or after this period. No doubt a significant problem has been to recognize the meager lamellar bone volumes added during short 1-2 week research periods in comparison to high degrees of normal interindividual gross morphological variation. The novel methods reported here are the first to provide quantitative results confirming the effects of microgravity with 24 hour resolution.

### Effect of Space Flight on Bone Density

Given our success at describing growth rate variability in the experimental rat, we undertook BSE-SEM of bone specimens in order to identify, with very high precision (i.e. 24 hours), differences in bone mineral density that may be dependent upon gravitational factors.

We provide preliminary results on flight specimens in Figure 5. The fluorescence and BSE-SEM images are provided at top. These images of the same field of view are brought to the same image size and orientation to allow for digital comparison. These images were processed together in order to confirm the duration of the experimental period on the BSE-SEM image. Relative bone density was recorded from within the rectangle on the BSE-SEM image.

The grey level profile from bottom to top, representing the beginning and end of the experimental period, is portrayed from right to left in the daily density profile below (dates are indicated above the relatively high density fractions associated with each bone lamella). High and low grey levels are associated with high and low mineral densities respectively. There is an initial variation in the density between the first calcein label and Launch. This may be an effect of the three vital labels administered during this time. Launch to Recovery is characterized by a broad downward trend in density at which point, after faltering once more, it rises. Toward the end of the experimental period, just one day prior to being euthanized, the relatively low density

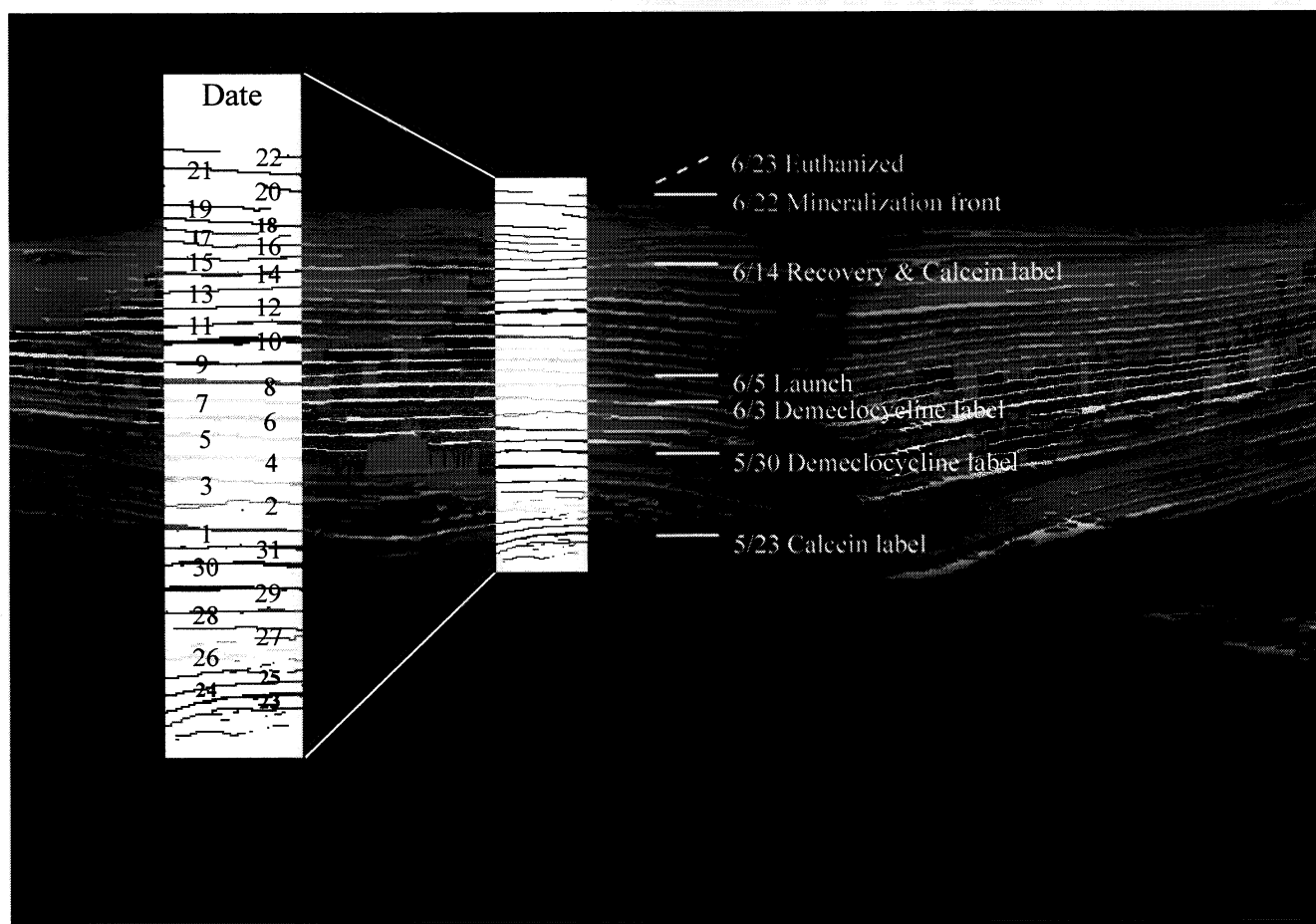
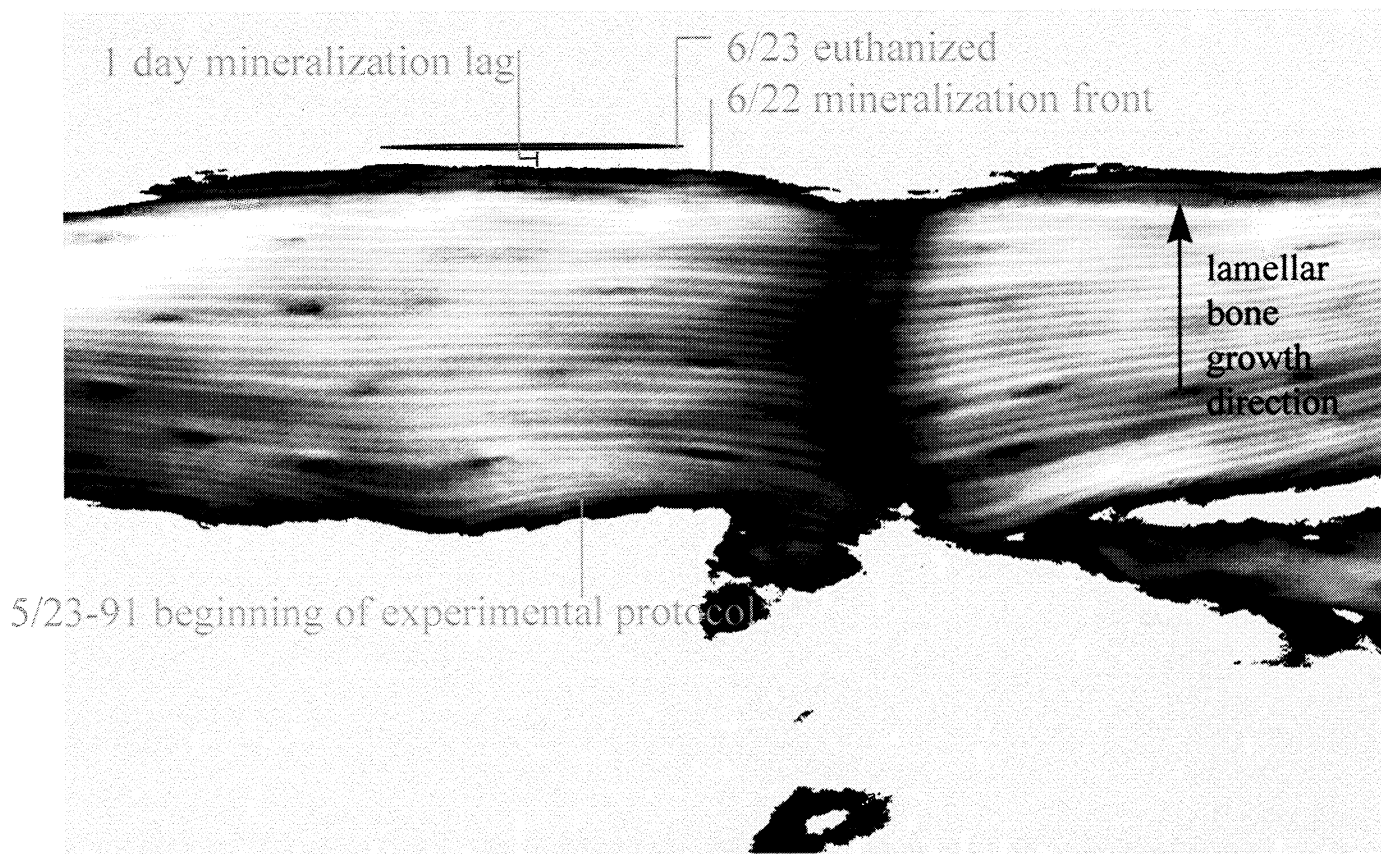
mineralization front is evident. This front is very recently formed bone which has still to acquire the higher mineralization levels of the lamellae behind it.

### YEAR 3

Year 2 work has enabled us to proceed with our anticipated Year 3 objectives which are to undertake quantitative investigations of lamellar growth rate variability of lamellar bone exposed to 2G and 3G, as well as the related backscattered electron microscopy.

Specimens still lagging in preparation and/or analysis will be processed, and all light and scanning electron microscopy will be completed. Acquisition of automatic staging this stage in Year 3 will permit us to make comparisons between LM and BSE-SEM results over significant bone volumes (the S440 SEM has an automatic stage). We also expect to refine the quantitative processing to permit the digital marrying of results of bone growth rate variability *and* density into a single quantitative portrayal of the bone growth and mineralization deficit during space flight with 24 hour resolution.

The quantitative results of our investigations on the morphological and mineral density changes affecting the development of lamellae under conditions of micro- and macrogravity will be summarized, written up in more final form for publication, and for presentation to the annual meeting of the *American Society for Gravitational and Space Biology*. A final project report will be written and presented to NASA scientists and officials.

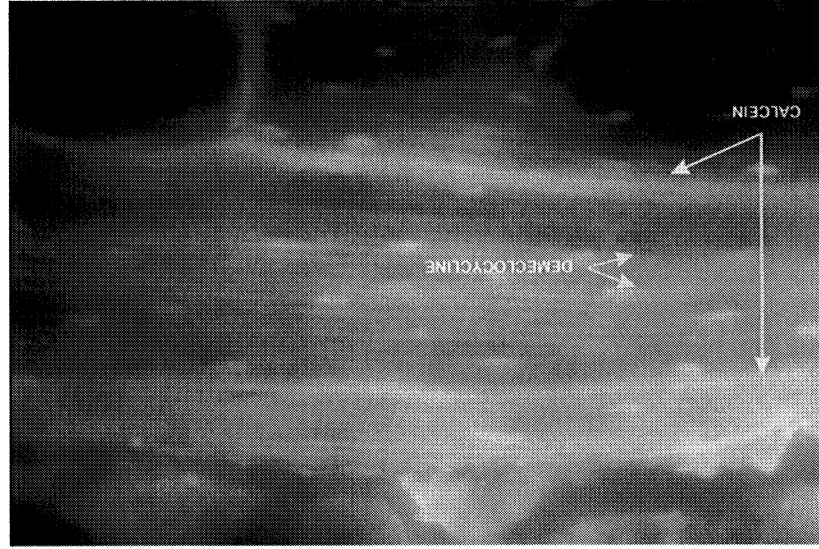


Experimental protocol

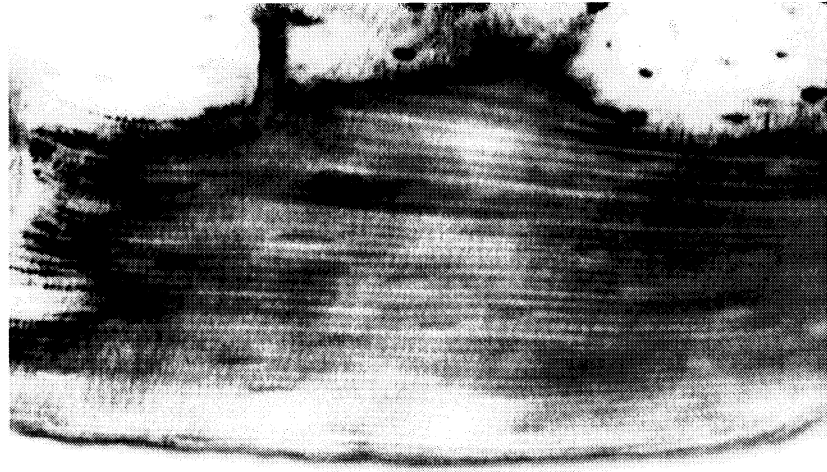
Figure 1

Flight

FLUORESCENCE IMAGE



POLARIZED LIGHT IMAGE



LAMELLAR PROCESSING

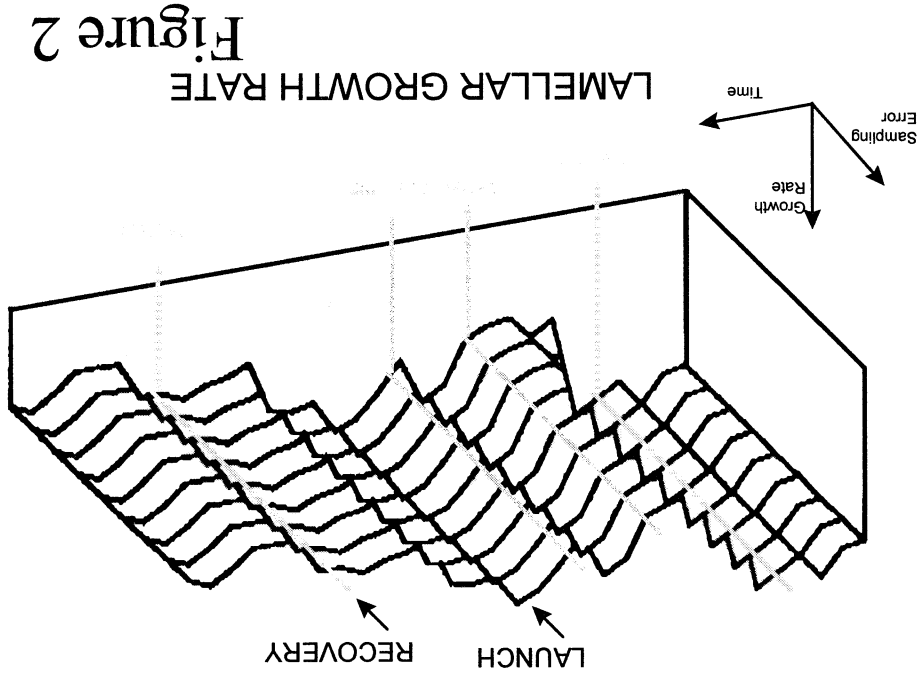
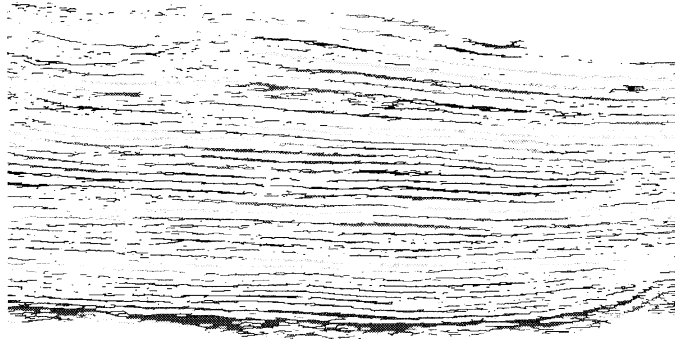
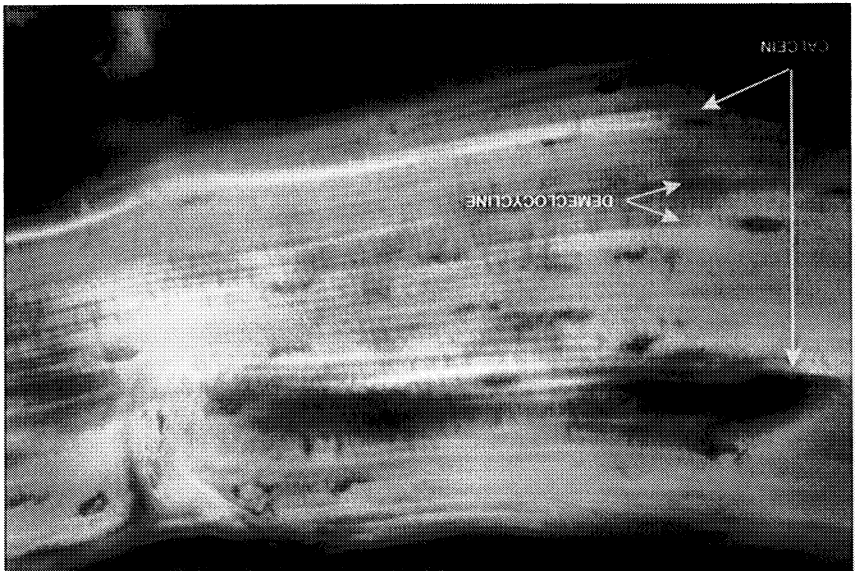


Figure 2



Control

FLUORESCENCE IMAGE

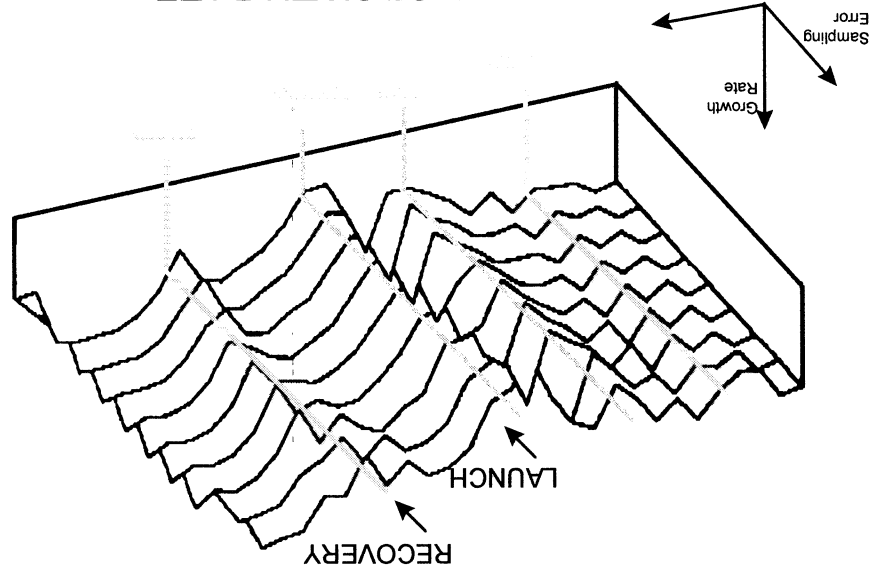


POLARIZED LIGHT IMAGE



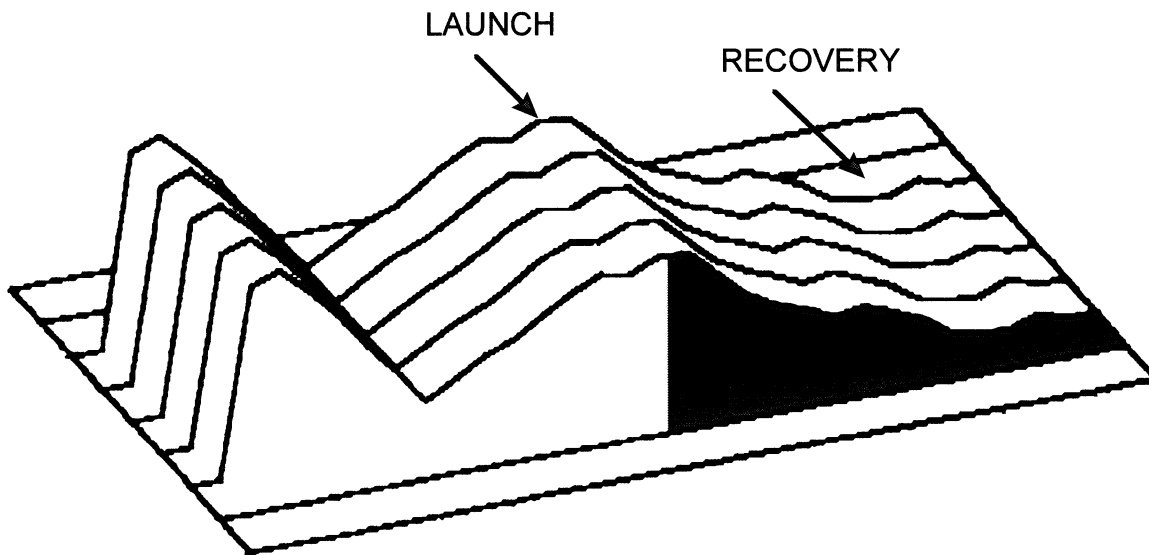
Figure 3

LAMELLAR GROWTH RATE

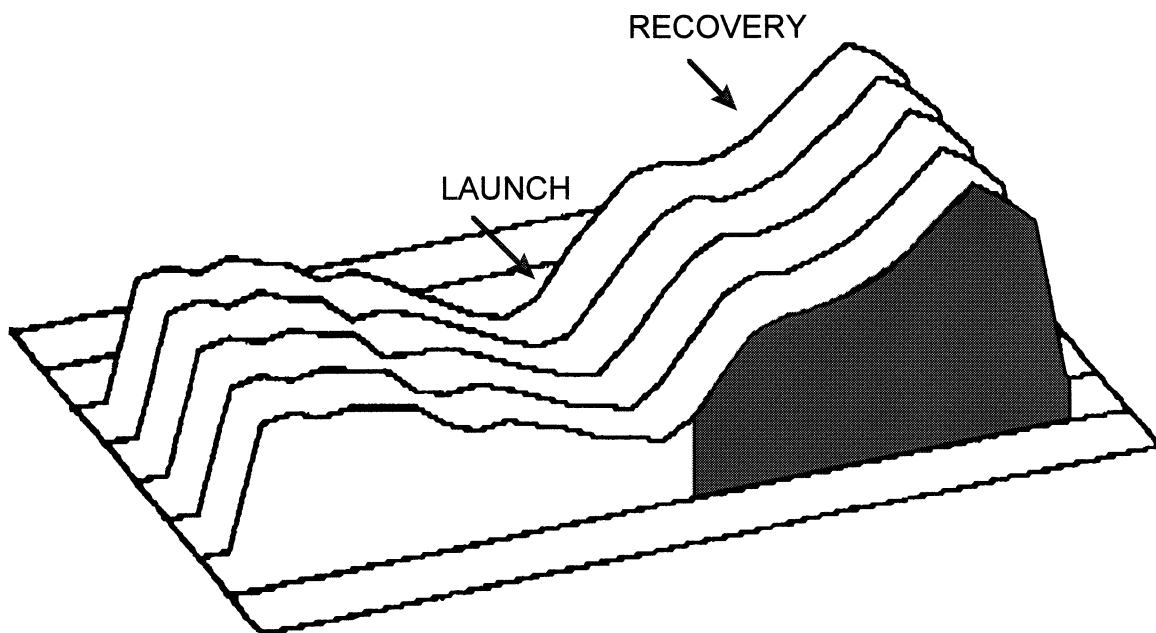


LAMELLAR PROCESSING





**FLIGHT:** Daily measures of in-flight bone development under conditions of microgravity (near zero G) reveal a decrease in the incremental growth rate.



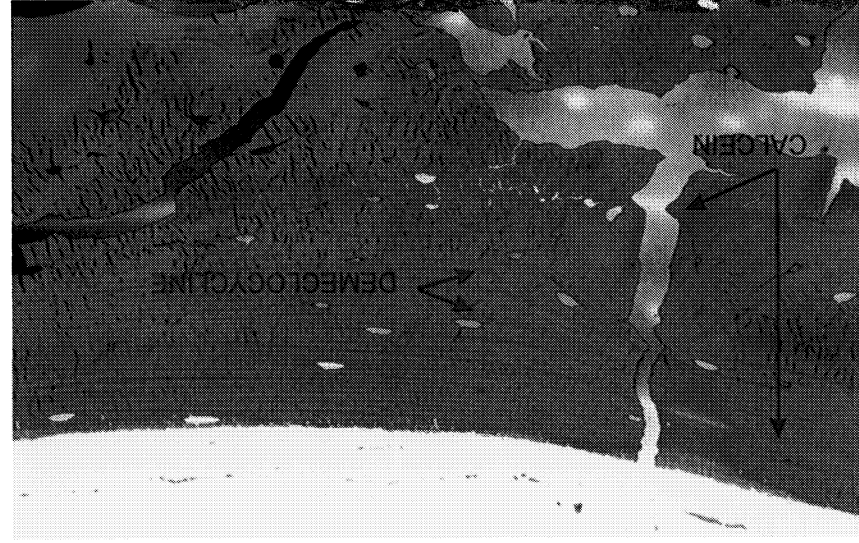
**CONTROL:** Daily measures of bone development under control conditions (one G) reveal a recovery in the incremental growth rate after vital label toxicity.

Bone Growth Rate

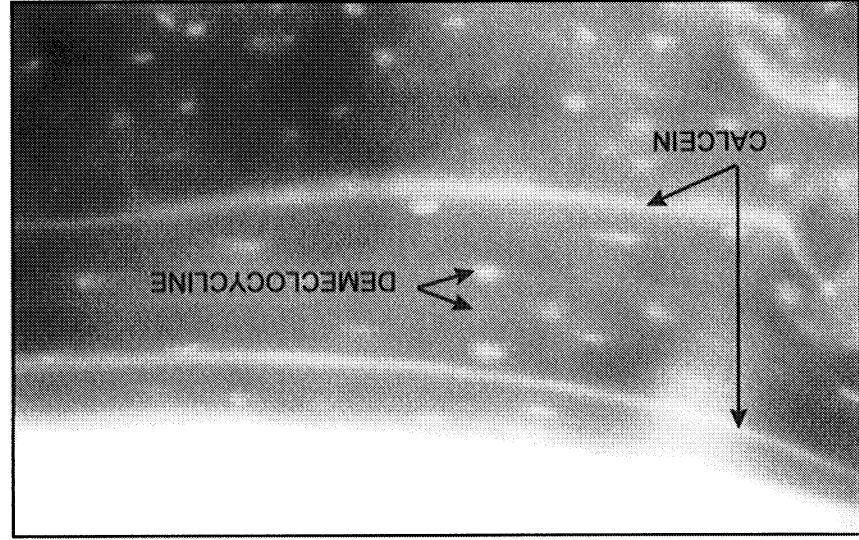
Figure 4

# Bone Density

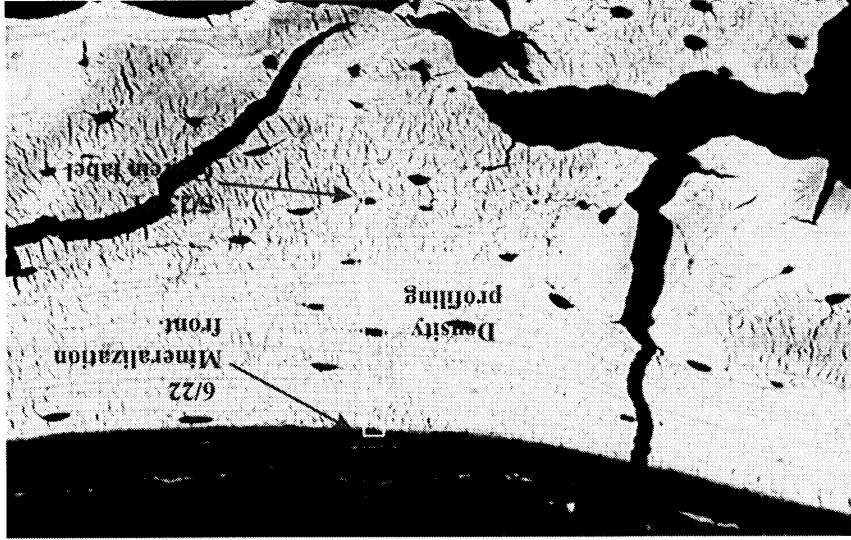
COMBINED PROCESSED IMAGE



FLUORESCENCE IMAGE



BSE-SEM IMAGE



DAILY DENSITY PROFILE

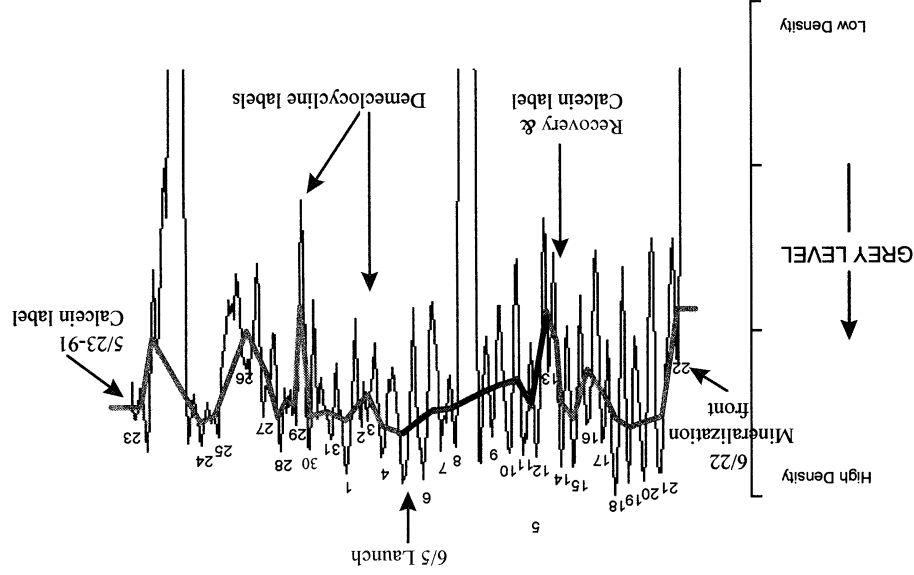


Figure 5